METHODS IN ENZYMOLOGY

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Murray P. Deutscher

DEPARTMENT OF BIOCHEMISTRY
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[33] One-Dimensional Gel Electrophoresis

By DAVID E. GARFIN

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) is an excellent method with which to identify and monitor proteins during purification and to assess the homogeneity of purified fractions. SDS–PAGE is routinely used for the estimation of protein subunit molecular weights and for determining the subunit compositions of purified proteins. SDS–PAGE can also be scaled up, for use in a preparative mode, to yield sufficient protein for further studies. In addition, two-dimensional analysis, combining isoelectric focusing with SDS–PAGE,[1,2] is a very high-resolution method for protein fractionation, enabling thousands of polypeptides to be resolved in a single gel. When used in conjunction with blotting methods,[3] SDS–PAGE provides one of the most powerful means available for protein analysis.

A great many electrophoretic systems have been developed and no attempt is made to summarize them here. In particular, the distinctions between the various "continuous" and "discontinuous" buffer systems are not discussed, nor are alternative support matrices considered. Gradient gels (gels whose pore sizes vary) are also omitted from discussion, since these can be prepared by relatively straightforward adaptation of any of a number of well-known methods for forming gradients. Rather, only the most common (and most reliable) analytical SDS–PAGE procedure[4] is described. Those wishing further information on the practical or theoretical aspects of electrophoretic processes can use Refs. 5 through 8 to gain access to the large volume of literature in the field. Some problems may require adoption of alternative procedures,[5-11] but for most applications the SDS–PAGE method presented here will perform satisfactorily.

[1] B. S. Dunbar, H. Kimura, and T. M. Timmons, this volume [34].
[2] B. S. Dunbar, "Two-Dimensional Electrophoresis and Immunological Techniques," Plenum, New York, 1987.
[3] T. M. Timmons and B. S. Dunbar, this volume [51].
[4] U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
[5] A. T. Andrews, "Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications," 2nd Ed. Oxford Univ. Press, New York, 1986.
[6] B. D. Hames, *in* "Gel Electrophoresis of Proteins: A Practical Approach" (B. D. Hames and D. Rickwood, eds.), p. 1. IRL Press, Oxford, 1981.
[7] R. C. Allen, C. A. Saravis, and H. R. Maurer, "Gel Electrophoresis and Isoelectric Focusing of Proteins: Selected Techniques." de Gruyter, Berlin, 1984.

Background

Although the detailed theory of gel electrophoresis is complicated and at present incomplete,[12-14] the fundamental concepts are easily understood. Briefly, in an electrophoretic separation, charged particles are caused to migrate toward the electrode of opposite sign under the influence of an externally applied electric field. The movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates for the constituent proteins of a sample.

In general, fractionation by gel electrophoresis is based on the sizes, shapes, and net charges of the macromolecules. Systems designed to fractionate proteins in their native configurations cannot distinguish between the effects of size, shape, and charge on electrophoretic mobility. As a consequence, proteins with differing molecular weights can have the same mobility in these systems. Thus, while PAGE methods for native proteins are valuable for separating and categorizing protein mixtures, they should not be used to assess the purity of a preparation or the molecular weight of an unknown.

SDS–PAGE overcomes the limitations of native PAGE by imposing uniform hydrodynamic and charge characteristics on all the proteins in a sample mixture. During sample preparation, proteins are treated with hot SDS. The anionic detergent binds tightly to most proteins at about 1.4 mg of SDS/mg of protein, imparting a negative charge to the resultant complexes.[15] Interaction with SDS disrupts all noncovalent protein bonds, causing the macromolecules to unfold. Concomitant treatment with a disulfide-reducing agent, such as 2-mercaptoethanol or dithiothreitol, further denatures proteins, breaking them down to their constituent subunits. The electrophoretic mobilities of the resultant detergent–polypeptide complexes all assume the same functional relationship to their molecular weights. Migration of SDS derivatives is toward the anode at

[8] A. Chrambach, "The Practice of Quantitative Gel Electrophoresis." VCH, Weinheim, 1985.

[9] P. J. Blackshear, this series, Vol. 104, p. 237.

[10] D. M. Neville, Jr., *J. Biol. Chem.* **246,** 6328 (1971); see also D. M. Neville and H. Glossmann, this series, Vol. 32, p. 92.

[11] A. F. Bury, *J. Chromatogr.* **213,** 491 (1981).

[12] T. M. Jovin, *Biochemistry* **12,** 871, 879, 890 (1973).

[13] A. Chrambach and T. M. Jovin, *Electrophoresis* **4,** 190 (1983).

[14] M. Bier, O. A. Palusinski, R. A. Mosher, and D. A. Saville, *Science* **219,** 1281 (1983).

[15] T. B. Nielsen and J. A. Reynolds, this series, Vol. 48, p. 3.

rates inversely proportional to the logarithms of their molecular weights.[10,16,17] SDS polypeptides, thus, move through gels in a predictable manner, with low-molecular-weight complexes migrating faster than larger ones. This means that the molecular weight of a protein can be estimated from its relative mobility in a calibrated SDS–PAGE gel and that a single band in such a gel is a criterion of purity.

Most electrophoresis is done in vertical chambers in gel slabs formed between two glass plates.[5,6] The slab format provides uniformity, so that different samples can be directly compared in the same gel. Gel thicknesses are established by spacers placed between the glass plates and sample wells are formed in the gels during polymerization with plastic, comb-shaped inserts. Electrophoresis cells provide means for sealing the assemblies during gel formation and for maintaining contact with the electrode buffers during runs. The better cells provide means for heat dissipation, because uneven heat distribution in the gel slab can cause band distortions.

Conventional gels are of the order of 16 to 20 cm long, 16 cm wide, and 0.5–3.0 mm in thickness and can accommodate about 25 samples. Thick gels have greater total protein capacity than thin ones, but are correspondingly less efficient at dissipating electrically generated heat and more difficult to stain and destain. Gel thicknesses of 0.75 or 1 mm are good compromise sizes, combining adequate protein loads and good staining speeds with minimal heat-related distortions. Typical runs take 4–5 hr.

Small-format cells (minicells) allow rapid analyses and are adequate for relatively uncomplicated samples. The design of these cells allows analyses to be completed two to three times faster than is possible with conventional cells. The gels are about 7 cm long × 8 cm wide and are very easily manipulated. Each gel can hold up to about 15 samples and a typical run can be completed in less than an hour (not counting set-up and polymerization time). The resolution of complex samples may be better in conventional gels than with minicells, since the separation of protein bands is improved by increasing the lengths of SDS–PAGE gels.

Polyacrylamide Gels

Polyacrylamide gels are formed by copolymerization of acrylamide monomer, $CH_2{=}CH{-}CO{-}NH_2$, and a cross-linking comonomer,

[16] A. L. Shapiro, E. Viñuela, and J. V. Maizel, Jr., *Biochem. Biophys. Res. Commun.* **28,** 815 (1967).

[17] K. Weber and M. Osborn, *J. Biol. Chem.* **244,** 4406 (1969).

N,N'-methylenebisacrylamide, $CH_2{=}CH{-}CO{-}NH{-}CH_2{-}NH{-}CO{-}CH{=}CH_2$, (bisacrylamide).[5-8,18] The mechanism of gel formation is vinyl addition polymerization and is catalyzed by a free radical-generating system composed of ammonium persulfate (the initiator) and an accelerator, tetramethylethylenediamine (TEMED). TEMED causes the formation of free radicals from persulfate and these in turn catalyze polymerization. Oxygen, a radical scavenger, interferes with polymerization, so that proper degassing to remove dissolved oxygen from acrylamide solutions is crucial for reproducible gel formation.

The sieving properties of a gel are established by the three-dimensional network of fibers and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains.[19] Within limits, as the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of protein molecules. By convention, a given gel is physically characterized by the pair of figures (*%T*, *%C*), where *%T* is the weight percentage of total monomer (acrylamide + cross-linker, in grams per 100 ml), and *%C* is the proportion of cross-linker (as a percentage of total monomer) in the gel. The practical limits for *%T* lie between 3 and 30%. The factors governing pore size are complicated, but, in general, the pore size of a gel decreases as *%T* increases. For any given fixed *%T*, pore size is at a minimum at about 5% *C*, increasing at both higher and lower cross-linker concentrations.[5-8,18]

The use of high-quality reagents is a prerequisite for reproducible, high-resolution gels. This is particularly true of acrylamide, which constitutes the most abundant component in the gel-monomer mixture. Residual acrylic acid, linear polyacrylamide, and ionic impurities are the major contaminants of acrylamide preparations. Moreover, buffer components should be of reagent grade and only distilled or deionized water should be used for all phases of gel electrophoresis.

In SDS–PAGE, the quality of the SDS is of prime importance. Differential protein-binding properties of impurities such as C_{10}, C_{14}, and C_{16} alkyl sulfates can cause single proteins to form multiple bands in gels.[20] Even with pure SDS, very basic proteins, very acidic proteins, various glycoproteins, and lipoproteins, because of their unusual compositions, migrate "anomalously" during electrophoresis.[5-7]

[18] A. Chrambach and D. Rodbard, *Science* **172**, 440 (1971).
[19] D. Rodbard and A. Chrambach, *Proc. Natl. Acad. Sci. U.S.A.* **65**, 970 (1970).
[20] M. M. Margulies and H. L. Tiffany, *Anal. Biochem.* **136**, 309 (1984).

Principle of Method

The most popular electrophoretic method is the SDS–PAGE system developed by Laemmli.[4-7,9] This is a discontinuous system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strength. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in better resolution than is possible using the same sample volumes in gels without stackers. Proteins, once concentrated in the stacking gel, are separated in the resolving gel.

The Laemmli SDS–PAGE system is made up of four components. From the top of the cell downward, these are the electrode buffer, the sample, the stacking gel, and the resolving gel. Samples prepared in low-conductivity buffer (0.06 *M* Tris-Cl, pH 6.8) are loaded between the higher conductivity electrode (0.025 *M* Tris, 0.192 *M* glycine, pH 8.3) and stacking gel (0.125 *M* Tris-Cl, pH 6.8) buffers. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear.[5-7,9,10,21] A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS–protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS–proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. (The stacking phenomenon is strikingly demonstrated with prestained protein standards, which are mixtures of proteins derivatized with reactive dyes.) The large-pore stacking gel (4% *T*) does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. (Proteins too large to enter the resolving gel will stop at the interface.) Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH (pH 9.5) formed by the Tris and glycine. Molecular sieving causes the SDS–polypeptide complexes to separate on the basis of their molecular weights.

[21] M. Wyckoff, D. Rodbard, and A. Chrambach, *Anal. Biochem.* **78**, 459 (1977).

Procedure

Equipment and reagents for SDS–PAGE can be obtained from a variety of suppliers. Electrophoresis cells vary in design, but their operation generally follows the steps outlined below. Since the many available cells differ in size, formulations are presented in conveniently sized units for simplicity. Required volumes can be prepared using multiples of these unit sizes. Except where noted, reagents for SDS–PAGE can be prepared as concentrated stock solutions.

Stock Solutions

Acrylamide concentrate (30% *T*, 2.7% *C*): Dissolve 29.2 g of acrylamide and 0.8 g of bisacrylamide in 70 ml of deionized water. When the acrylamide is completely dissolved, add water to a final volume of 100 ml. Filter the solution under vacuum through a 0.45-μm membrane. Store stock acrylamide at 4° in a dark bottle for no more than 1 month. *Caution:* Acrylamide monomer is a neurotoxin. Avoid breathing acrylamide dust, do not pipette acrylamide solutions by mouth, and wear gloves when handling acrylamide powder or solutions containing it. For disposal of unused acrylamide, add bisacrylamide (if none is present), induce polymerization, and discard the solidified gel

1.5 *M* Tris-Cl, pH 8.8, concentrated resolving gel buffer: Dissolve 18.2 g Tris base in ≈80 ml of water, adjust to pH 8.8 with HCl, and add water to a final volume of 100 ml. Store at 4°

0.5 *M* Tris-Cl, pH 6.8, concentrated stacking gel buffer: Dissolve 6.1 g Tris base in ≈80 ml of water, adjust to pH 6.8 with HCl, and add water to a final volume of 100 ml. Store at 4°

10% (w/v) sodium dodecyl sulfate (SDS): Dissolve 10 g SDS in ≈60 ml of water and add water to a final volume of 100 ml

Stock sample buffer (0.06 *M* Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.025% Bromphenol Blue):

Water	4.8 ml
0.5 *M* Tris-Cl, pH 6.8	1.2 ml
10% SDS	2.0 ml
Glycerol	1.0 ml
0.5% Bromphenol Blue (w/v water)	0.5 ml

Store at room temperature. SDS-reducing buffer is prepared by adding 50 μl of 2-mercaptoethanol to each 0.95 ml of stock sample buffer before use

Catalyst

10% ammonium persulfate (APS): Dissolve 100 mg APS in 1 ml of water. Make the APS solution fresh daily

TEMED (*N,N,N′,N′*-tetramethylethylenediamine): Use TEMED undiluted from the bottle. Store cool, dry, and protected from light

Electrode Buffer

Electrode buffer: 0.025 *M* Tris, 0.192 *M* glycine, 0.1% (w/v) SDS, pH 8.3 (0.3 g Tris base, 1.4 g glycine, 1 ml 10% SDS/100 ml electrode buffer). Do not adjust the pH of the electrode buffer; just mix the reagents together and confirm that the pH is near 8.3 (±0.2). Electrode buffer can be made as a 5× concentrate consisting of 15 g Tris base, 72 g glycine, and 5 g SDS/liter. 5× electrode buffer concentrate must be stored in glass containers. To use 5× concentrate, dilute it with four parts water

Casting Gels

Thoroughly clean the glass plates, spacers, combs, and upper buffer reservoir of the gel apparatus with detergent and rinse them well. Wear gloves while assembling the equipment. The resolving gel is cast first, then overlaid with the stacking gel.

1. Assemble the casting apparatus and determine the gel volume from the manufacturer's instructions or by calculation. A 1- to 2-cm stacking gel is used above the resolving gel. Determine the height to which the resolving gel is to be poured by inserting a well-forming comb between the glass plates and marking the outer plate 1–2 cm below the teeth of the comb.

2. Prepare the monomer solution for the appropriate resolving gel by combining all of the reagents in Table I except the ammonium persulfate (APS) and TEMED; a disposable, plastic beaker is a convenient mixing vessel. The two gel recipes given in Table I cover the molecular weight ranges usually encountered. Gels of any other acrylamide concentration desired[5,6,9] can be prepared by adjusting (only) the amounts of 30% monomer stock and water used in the recipes. Deaerate the solution under vacuum (e.g., in a bell jar or desiccator) for at least 15 min.

3. Gently mix the APS and TEMED (Table I) into the deaerated monomer solution. Using a pipet and bulb, add the monomer solution between the gel plates up to the mark delimiting the resolving gel. Immediately overlay the monomer solution with water-saturated 2-butanol or

TABLE I
FORMULATIONS OF SDS–PAGE RESOLVING GELS[a]

Component	7.5% T[b]	12% T[c]
Water	4.85 ml	3.35 ml
1.5 *M* Tris-Cl, pH 8.8	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
Acrylamide/bis (30% *T*, 2.7% *C*)	2.5 ml	4.0 ml
10% ammonium persulfate[d]	50 μl	50 μl (0.05%)
TEMED	5 μl	5 μl (0.05%)

[a] Any desired volume of monomer solution can be prepared by using multiples of the 10-ml recipes. Combine the first four items and deaerate the solution under vacuum for 15 min. Start polymerization by adding ammonium persulfate and TEMED.

[b] For SDS-treated proteins in the approximate molecular weight range between 40K and 250K.

[c] For SDS-treated proteins in the approximate molecular weight range between 10K and 100K.

[d] To make 10% ammonium persulfate (APS), dissolve 100 mg APS in 1 ml of water. Make the APS solution fresh daily.

tert-amyl alcohol to exclude air, which might inhibit polymerization, from the surface of the monomer mixture. Allow the gel to polymerize for 45 min to 1 hr. Polymerization is evidenced by the appearance of a sharp interface beneath the overlay, which will start to become visible in about 15 min. Polymerization is essentially complete in about 90 min, but the stacking gel can be poured after about an hour.[22] Allow unused monomer to polymerize in the beaker and discard the gel.

4. Prepare 10 ml of stacking gel monomer solution (4% *T*, 2.7% *C*), by combining

Water	6.1 ml
0.5 *M* Tris-Cl, pH 6.8	2.5 ml
Acrylamide stock solution (30% *T*)	1.3 ml
10% SDS	0.1 ml

Deaerate the monomer solution under vacuum for at least 15 min.

5. Thoroughly rinse the top of the resolving gel with water and dry the area above it with filter paper. Place a well-forming comb between the gel plates and tilt it at a slight angle to provide a way for bubbles to escape.

[22] Bio-Rad Lab., Bull. No. 1156.

6. Add 50 μl of 10% APS and 10 μl of TEMED to each 10 ml of degassed monomer solution and pour the stacking gel solution on top of the resolving gel. Align the comb in its proper position, being careful not to trap bubbles under the teeth. Visible polymerization of the stacking gel should occur in about 10 min. No overlay is required, because the comb excludes oxygen from the surfaces of the wells. Allow the gel to polymerize for 30–45 min. Allow unused monomer to polymerize in the beaker before disposing of it.

In some situations, it may be necessary or convenient to let the gel stand overnight before it is used. When this is the case, it is best to pour the stacking gel on the day of the run to maintain the ion discontinuities at the interface between the two gels. For storage, the top of the resolving gel should be rinsed thoroughly and covered with resolving gel buffer (0.375 *M* Tris-Cl, 0.1% SDS, pH 8.8) to avoid dehydration and ion depletion. Also, the tops of the gel sandwiches should be covered with plastic wrap during storage.

Sample Preparation

The common biochemical buffers are usually tolerated in SDS–PAGE, so that pretreatment of samples is not generally required. Distorted band patterns, such as pinching or flaring of lanes, can be caused by excessive amounts of salt in the samples. These distortions can often be remedied by desalting the samples.

1. Prepare the volume of SDS-reducing buffer required for the number of samples to be run by adding 50 μl of 2-mercaptoethanol to each 0.95 ml of stock sample buffer (to a final concentration of 5% 2-mercaptoethanol). This step may be omitted, if reduction of disulfide bonds is not desired.

2. Dilute samples with at least 4 vol of complete SDS-reducing buffer (although as little as 2-fold dilution may be adequate for some samples). Sample volumes are of the order of 20–50 μl for conventional gels and 5–30 μl for minicells, depending on the widths of the wells and the thicknesses of the gels. Detection in gels requires on the order of 1 μg of protein per band for easy visibility when staining with Coomassie Blue R-250 or 0.1 μg of protein per band with silver staining (see below).

3. Heat the diluted samples at 95° for 4 min by suspending the sample tubes in hot water. Do not store prepared samples.

Electrophoresis

Assemble the electrophoresis cell, fill the upper and lower reservoirs with electrode buffer, and remove the comb from the stacking gel. Load

the prepared samples into the wells in the stacking gel by layering them under electrode buffer using a microliter syringe or micropipet. The glycerol in the samples provides the necessary density for them to sink to the bottoms of the wells and the Bromphenol Blue tracking dye enables the samples to be seen during loading. Finally, attach leads to the unit and connect them to a power supply. The lower electrode is the anode and the upper one is the cathode, in SDS–PAGE.

During an electrophoresis run, electrical energy is converted to heat which can cause band distortion and diffusion. In general, electrophoresis should be carried out at power settings at which the run proceeds as rapidly as the chamber's ability to draw off heat will allow. In other words, the run should be as fast as possible without exceeding desired resolution and distortion limits.

Many of the power supplies which are available allow control of any electrical quantity and the choice is almost a matter of preference. Constant current conditions, as a rule, result in shorter but hotter runs than does constant voltage.[7] In the early stages of a run, the resistance of the gel increases as the chloride ions migrate out of it. Accordingly, voltage will rise or current will fall, depending on whether constant current or constant voltage operation is in use.

Small-format minicells, with their thin glass plates, are better able to efficiently dissipate the heat generated by the initially high currents at the beginnings of runs than are standard-sized cells. Thus, the recommendation is that gels should be run under constant current conditions (16–24 mA/mm of gel thickness) in conventional apparatus and at constant voltage (20–30 V/cm of gel length) in minicells. The use of recirculated coolant, where possible, allows higher voltages and currents to be used for shortened run times. Electrophoresis should be started immediately after the samples are loaded and is generally continued until the Bromphenol Blue tracking dye has reached the bottom of the gel.

Comments on Method

The Laemmli SDS–PAGE system[4–7,9] is an adaptation of an earlier method devised by Ornstein[23] and Davis[24] for fractionation of native serum proteins. The different (discontinuous) buffers used in the stacking and resolving gels are required for the proper functioning of the Ornstein–Davis system.[12,23] However, inclusion of SDS modifies the rationale of the Ornstein–Davis technique in important ways, since the properties of the detergent dominate the system.[7,8,21]

[23] L. Ornstein, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964).
[24] B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).

The necessary components of the Laemmli SDS–PAGE system are a Tris-Cl gel buffer, the Tris–glycine–SDS electrode buffer, and the SDS-reducing sample buffer. As a consequence of SDS in the system, it is actually not necessary to cast the stacking gels at different pH or ionic strength than the resolving gels. Similar resolution is obtained whether the stacking gel is cast as above or in resolving gel buffer (0.375 *M* Tris-Cl, pH 8.8). This is because the mobilities of SDS–polypeptide complexes are insensitive to pH in this range.[7] When many gels are being cast at one time for storage and later use, it is convenient to cast the stacking and resolving gels in the same buffer.

Total SDS load, on the other hand, has considerable influence on resolution.[21] Inclusion of more than 200 μg of SDS in 30- to 50μl samples in the minigel configuration can lead to broadening and spreading of protein bands. With dilute, large volume samples, it may prove advantageous to limit the total SDS in the system by dropping the final SDS concentration of the treated sample to about 0.5% and casting the gels without SDS. Because the mobility of SDS is greater than those of proteins, SDS from the electrode buffer quickly overtakes the proteins during electrophoresis. The gel is thus supplied and continuously replenished with SDS from the electrode buffer at a level sufficient to maintain the saturation of the proteins.[8]

Variations of Method

The complete denaturation and dissociation of proteins with the Laemmli SDS–PAGE system[4–7,9] are not always desirable. For some analyses, it might be of interest to estimate the molecular weights of particular proteins in their intact, oligomeric forms. In other experiments, interest might center on the biological activities of proteins in their native, nondenatured states. Through selective use of the two denaturants, 2-mercaptoethanol and SDS, conditions can be adjusted as needed to separate proteins in the completely denatured, partially denatured, or native states.

Covalent associations between protein units can be maintained by omitting 2-mercaptoethanol from the sample buffer. In the absence of the reducing agent, the intra- and interchain disulfide bonds of sample proteins remain intact. The electrophoretic mobilities of the resultant SDS-protein complexes are correspondingly altered relative to those obtained under dissociating conditions. During electrophoresis, the mobilities of oligomeric SDS-proteins are lower than those of their fully denatured SSDS-polypeptide components. Further, the electrophoretic behaviors of single-chain polypeptides can also be affected by reduction. The intra-

chain disulfide bridges of single-chain proteins can hold them in compact configurations that are more or less retained in the presence of SDS. Thus, some SDS-proteins migrate faster electrophoretically in the absence of 2-mercaptoethanol than when in the extended structures brought on by reduction. Proteins often show characteristic, individual responses to reduction, so that comparisons of SDS–PAGE gels run with and without 2-mercaptoethanol can be very informative.[25]

To separate proteins without reduction, carry out the SDS–PAGE procedure described above, omitting 2-mercaptoethanol from the sample buffer. Note that oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. It may, therefore, be necessary to use lower concentration (%*T*) gels than with the fully denaturing method to get oligomers to move adequate distances into the matrices. In addition, nonreduced proteins may not be completed saturated with SDS and, hence, may not bind the detergent in a constant weight ratio. This makes molecular weight determinations of these molecules by SDS–PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

When both SDS and 2-mercaptoethanol are left out of the Laemmli procedure, what remains is the classical Ornstein–Davis PAGE system[23,24] for native proteins. This is a high-resolution native PAGE method designed for separation of the full spectrum of serum proteins. Because the system was meant to separate a wide variety of proteins, resolution may not be optimal for some restricted ranges of protein mobilities. Although there are a number of high-resolution native PAGE systems available to meet differing requirements,[5–9] the Ornstein–Davis method should perform adequately for the fractionation of the majority of commonly encountered protein mixtures. Molecular weights are more difficult to determine by native PAGE than by SDS–PAGE, since a single native system cannot distinguish the effects of charge and conformation on protein electrophoretic mobilities.[5–8]

The procedure described here is readily modified for native PAGE. Merely omit 2-mercaptoethanol from the sample buffer and replace the 10% SDS in the recipes for the gel, sample, and electrode buffers with equivalent volumes of water. Follow the procedure as otherwise presented, except for sample treatment. Samples should be diluted in nondenaturing buffer (0.06 *M* Tris-Cl, pH 6.8, 10% glycerol, 0.025% Bromphenol Blue) following the same guidelines as for denaturing gels, but they should not be heated.

[25] T. Marshall, *Clin. Chem.* **30**, 475 (1984).

Detection of Proteins in Gels

Three of the simplest and most reliable methods for the detection of proteins in SDS–PAGE gels are presented. They should be adequate to cover the requirements of most situations. Coomassie Brilliant Blue R-250 is the most common protein stain and is recommended for routine work. Silver staining is the most sensitive method for staining proteins in gels and should be employed when electrophoresis is used to assess the purity of a preparation; e.g., an antigen preparation. Copper staining is a recent development allowing rapid and sensitive staining. Discussions of other detection methods, including radiolabeling and means for quantitating proteins in gels, can be found in Refs. 2, 5–7, and 26.

After electrophoresis, remove the gel assembly and separate the glass plates. The gel will probably stick to one of the two plates. Remove the spacers and cut off and discard the stacking gel. Place the glass plate holding the gel into fixative or staining solution and float the gel off of the plate. All of the steps in gel staining are done at room temperature with gentle agitation (e.g., on an orbital shaker platform) in any convenient container, such as a glass casserole or a photography tray. Always wear gloves when staining gels, since fingerprints will stain. Permanent records of stained gels can be obtained by photographing them or by drying them on filter paper using commercially available drying apparatus.

Dye Staining with Coomassie Brilliant Blue R-250

This is the standard method of protein detection.[5–7,27] Easy visibility requires on the order of 0.1–1 μg of protein per band.

1. Prepare the staining solution: 0.1% Coomassie Brilliant Blue R-250 (w/v) in 40% methanol (v/v), 10% acetic acid (v/v). Filter the staining solution after the dye has dissolved. The staining solution is reusable. Store it at room temperature.
2. Soak the gel in an excess of staining solution for 30 min.
3. Destain with a large excess of 40% methanol, 10% acetic acid. Change the destaining solution several times, until the background has been satisfactorily removed.

The acid–alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-weight proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by incubating the gel in 40% methanol (v/v), 10%

[26] C. R. Merril, this volume [36].
[27] C. M. Wilson, this series, Vol. 91, p. 236.

trichloroacetic acid (w/v) for 1 hr before it is immersed in the staining solution.

Silver Staining

This method, developed by Merril and co-workers, can be as much as 100 times more sensitive than dye staining.[7,28] Bands containing 10–100 ng of protein can be easily seen. The reagents are available in kit form from Bio-Rad Laboratories.

Reaction times vary with the thicknesses of the gels.

1. Fix the proteins in the gel in about 400 ml of 40% methanol, 10% acetic acid (v/v) (or 40% methanol, 10% trichloroacetic acid) for 30 min to overnight.
2. Fix twice in 400 ml 10% ethanol, 5% acetic acid (v/v) for 15–30 min.
3. Soak the gel for 3–10 min in 200 ml of fresh oxidizer solution (0.0034 *M* potassium dichromate, 0.0032 *N* nitric acid).
4. Wash the gel three or four times for 5–10 min in 400 ml water, until the yellow color has been washed out.
5. Soak the gel in 200 ml fresh silver reagent (0.012 *M* silver nitrate) for 15–30 min.
6. Wash the gel with 400 ml water for 1–2 min.
7. Wash the gel for about 1 min in developer (0.28 *M* sodium carbonate, 1.85% paraformaldehyde).
8. Replace the developer with fresh solution and incubate for 5 min.
9. Replace the developer a second time and allow development to continue until satisfactory staining has been obtained.
10. Stop development with 5% acetic acid (v/v).

Vertical streaks and sample-independent bands in the 50- to 70-kDa region are sometimes seen in silver-stained gels. These artifacts have been attributed to reduction of contaminants inadvertently introduced into the samples.[29] They can be eliminated by adding excess iodoacetamide to sample solutions after treatment with SDS-reducing buffer.[30]

Copper Staining

Rapid, single-step staining of SDS–PAGE gels is achieved by incubating gels in copper chloride.[31] The resultant, negatively stained image of the electrophoretogram is intermediate in sensitivity between Coomassie blue and silver staining.

1. Wash the gel briefly in water.
2. Soak the gel in 0.3 *M* $CuCl_2$ for 5 min.
3. Wash the gel for 2–3 min in water.

The method yields negatively stained gels showing clear protein bands on an opaque, blue–green background. The protein bands can be easily seen and photographed with the gel on a black surface. Proteins are not permanently fixed by this method and can be quantitatively eluted after chelating the copper.[31] The electrophoretic pattern is lost when copper-stained gels are dried so they must be photographed, restained with Coomassie Blue, or stored in water.

Marker Proteins

Mixtures of marker proteins are available for calibrating gels. PAGE standards are mixtures of proteins with precisely known molecular weights blended for uniform staining. They are obtainable in various molecular weight ranges. Concentrated stock solutions of the standards are diluted in sample buffer just prior to electrophoresis and treated in the same manner as the sample proteins. These proteins are suitable as reference markers for molecular weight determinations.

Prestained SDS–PAGE standards have recently become available. The coupling of dye molecules to the marker proteins changes their molecular weights significantly and unpredictably and they should not be used for molecular weight determinations. However, prestained standards are very useful for following the course of an electrophoretic run and are valuable for assessing the efficiencies of protein transfers when gels are blotted.

Molecular Weight Determination

Molecular weights of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight.[5–9] After the gel has been run, but before it has been stained, mark the position of the Bromphenol Blue tracking dye to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in india ink into the gel at the dye front. After staining, measure the migration distances of each protein (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and

[28] C. R. Merril, D. Goldman, S. A. Sedman, and M. H. Ebert, *Science* **211**, 1437 (1981); see also C. R. Merril, D. Goldman, and M. L. Van Keuren, this series, Vol. 104, p. 441.

[29] D. Ochs, *Anal. Biochem.* **135**, 470 (1983).

[30] A. Görg, W. Postel, J. Weser, S. Günther, J. R. Strahler, S. M. Hanash, and L. Somerlot, *Electrophoresis* **8**, 122 (1987).

[31] C. Lee, A. Levin, and D. Branton, *Anal. Biochem.* **166**, 308 (1987).

conventionally denoted as R_f. Construct a (semilogarithmic) plot of the logarithms of the molecular weights of the protein standards as functions of the R_f values. Note that the graphs are slightly sigmoid. As long as the extremities of a molecular weight range are avoided, unknown molecular weights can be estimated by linear regression analysis or interpolation from the curves of log M_r vs R_f. Keep in mind that the molecular weights obtained using SDS–PAGE are those of the polypeptide subunits and not those of native, oligomeric proteins.

Preparative Electrophoresis

The most satisfactory way to recover proteins separated by SDS–PAGE for further study is to extract them from bands excised from the gels. Many attempts have been made to design continuous elution devices suitable for routine protein purification, in which bands emerging from the bottoms of electrophoresis gels are swept away to fraction collectors.[5,8,32] The scarcity of preparative gel devices is evidence of the disappointing lack of success in developing generally useful instruments. Preparative gel electrophoresis would ideally be capable of yielding high-milligram to gram quantities of individual proteins recovered cleanly with the resolution anticipated from the corresponding analytical gels. In general, though, band distortion and poor elution have limited the resolution attainable with most apparatus so that they have only worked well with relatively simple protein mixtures. The difficulties in scaling gel electrophoresis up to preparative levels has tended to result in devices which are rather cumbersome and which require much technical skill for best results. As a consequence, proteins are usually obtained by extraction from analytical type gels.[33]

Gels to be run for the isolation of proteins[5,8] can be cast using special preparative combs. These combs form wide sample wells spanning the widths of the gels and usually provide a separate, narrow reference well for marker proteins. The maximum amount of sample which can be loaded on a gel ultimately depends on how well the proteins of interest are separated from their neighbors in the sample mixture. Since bands become wider as the amount of material increases, as sample load is raised, the corresponding loss of resolution will eventually become unacceptable. Protein loads 10- to 50-fold greater per unit of cross-sectional area than are usually run in analytical gels are easily tolerated. Thus, with some large slab gels, proteins can be recovered in tens-of-milligram amounts.

[32] A. Chrambach and N. Y. Nguyen, *in* "Electrokinetic Separation Methods" (P. G. Righetti, C. J. Van Oss, and J. W. Vanderhoff, eds.), p. 337. Elsevier, Amsterdam, 1979.
[33] M. Harrington, this volume [37].

Copper staining[31] (above) is advisable for the visualization of the bands in preparative SDS–PAGE, since this method does not employ fixative solvents. Desired bands are cut from the gel and destained by incubation in three changes (for 10 min each) of 0.25 *M* EDTA, 0.25 *M* Tris-Cl, pH 9. After destaining, gel slices are incubated in the appropriate elution buffer.

Proteins are often extracted from macerated gel slices by simple diffusion into appropriate buffers or by solubilization of the gel.[5,33] In the latter method, cross-linkers other than bisacrylamide are copolymerized into the gels.[5,7] For example, gels cross-linked with *N,N'*-bisacrylylcystamine (BAC) are dissolvable in 2-mercaptoethanol or dithiothreitol, while both *N,N'*-dihydroxyethylenebisacrylamide (DHEBA) and *N,N'*-diallyltartardiamide (DATD) result in gels which can be solubilized with periodic acid. Once gels have been dissolved, proteins must be separated from the large excess of gel material by gel filtration or ion-exchange chromatography.

Electrophoretic elution is an efficient method for recovering proteins from gel slices.[2,5,8] In the simplest versions of this method, proteins are electrophoresed out of gel pieces into dialysis sacks in the types of apparatus used for running cylindrical gel rods. Devices are available for the rapid recovery of proteins in small volumes with yields of greater than 70% in most cases. Elution takes about 3 hr at 10 mA/tube in 0.025 *M* Tris, 0.192 *M* glycine, 0.1% SDS, pH 8.3 (standard SDS–PAGE electrode buffer). SDS can be removed from the eluted samples by dialysis or ion-exchange chromatography.[34]

[34] A. J. Furth, *Anal. Biochem.* **109**, 207 (1980).

[34] Protein Analysis Using High-Resolution Two-Dimensional Polyacrylamide Gel Electrophoresis

By BONNIE S. DUNBAR, HITOMI KIMURA, and THERESE M. TIMMONS

The term two-dimensional electrophoresis has been used to describe a variety of methods employing separation of molecules in two dimensions. The term high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is now specifically applied to the separation of proteins in the first dimension according to their isoelectric points using isoelectric focusing (IEF) with carrier ampholytes after reduction of disulfide bonds,